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TITLE: Mouse Model of Human Hereditary Pancreatitis

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14. ABSTRACT The aim of our research is to generate and characterize mouse models of human hereditary pancreatitis that develop pancreatitis spontaneously or exhibit increased sensitivity to experimentally induced pancreatitis. Such models are desperately needed to study in vivo the mechanistic aspects of the trypsin-dependent pathway in pancreatitis and to begin testing therapeutic and preventive approaches. Mutations in the digestive enzyme trypsinogen cause hereditary pancreatitis in humans. Previous attempts to introduce these mutant forms of human trypsinogen into mice have failed to produce models that recapitulate the human disease. Therefore, we introduced mutations in the endogenous mouse T7 cationic trypsinogen gene and obtained several new strains carrying mutations D23A, D23del or K24R. Remarkably, mice with the D23A mutation develop spontaneous, progressive pancreatitis with eventual destruction and fatty replacement of the exocrine pancreas and preservation of endocrine tissue. On the other hand, mouse strains carrying the D23del or K24R mutations do not develop spontaneous pancreatitis but may be more susceptible to experimentally induced pancreatitis. These novel models will afford important insight into the role of trypsin in human hereditary pancreatitis and facilitate testing of trypsin inhibition as a therapeutic approach.					
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1.INTRODUCTION: The aim of our research is to generate and characterize mouse models of human hereditary pancreatitis that develop pancreatitis spontaneously or exhibit increased sensitivity to experimentally induced pancreatitis. Such a mouse model is desperately needed to study *in vivo* the mechanistic aspects of the trypsin-dependent pathway in pancreatitis and to begin testing therapeutic and preventive approaches. To this end, we introduce mutations in the endogenous mouse T7 cationic trypsinogen, knocked-in to the T7 locus. The newly created mouse strains are then characterized with respect to spontaneous pancreatitis and sensitivity to experimentally induced pancreatitis.

2.KEYWORDS: chronic pancreatitis, acute pancreatitis, trypsinogen mutation, trypsinogen activation, trypsin, knock-in mouse model, experimental pancreatitis

3.ACCOMPLISHMENTS:

What were the major goals of the project? Our original proposal had three specific aims.

Aim 1. Identify and biochemically characterize mutations that phenocopy the effect of human hereditary-pancreatitis associated mutations in mouse trypsinogen isoform T7.

Aim 2. Generate knock-in strains with mutated T7 trypsinogen carrying mutations in the C57BL/6 background.

Aim 3. Characterize the newly created mouse strains with respect to spontaneous pancreatitis and sensitivity to experimentally induced pancreatitis.

What was accomplished under these goals?

Aim 1 has been completed; Aim 2 has been partially completed; and significant progress has been made towards the completion of Aim 3. The following goals have been accomplished:

Specific Aim 1. Identify and biochemically characterize mutations that phenocopy the effect of human hereditary-pancreatitis associated mutations in mouse trypsinogen isoform T7.

Under this aim, we had two major tasks in our SOW: Major Task 1 was the design and construction of mutant forms of the T7 mouse trypsinogen gene and expression and purification of these mutant enzymes. Major Task 2 was to analyze autoactivation of the T7 trypsinogen mutants to determine which mutants might be appropriate for the generation of knock-in mice.

THIS AIM HAS BEEN COMPLETED. ALL STATED GOALS HAVE BEEN MET. See our previous year-1 report for detailed description of accomplishments.

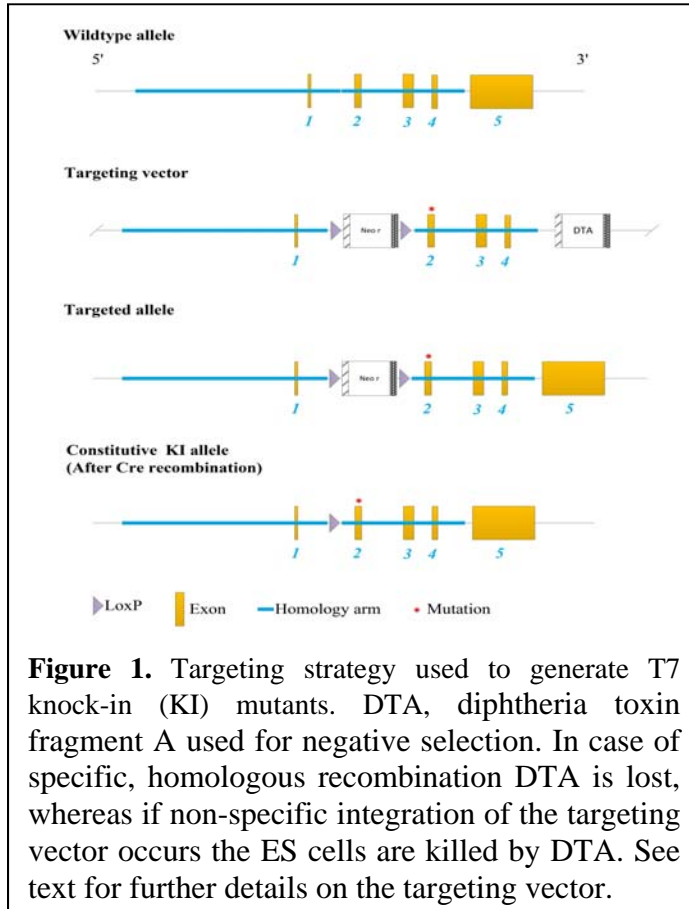
SUMMARY OF RESULTS FOR SPECIFIC AIM 1. We successfully identified mutations which can phenocopy the effect of human hereditary-pancreatitis-associated mutations in the context of the mouse T7 trypsinogen. Thus, these mutations all increase autoactivation of mouse T7 trypsinogen to various extents. In the next aim, we built on these data and generated knock-in mouse strains carrying some of these mutations.

Specific Aim 2. Generate knock-in mouse strains with mutated T7 trypsinogen in the C57BL/6 background.

Under this aim, we had two major tasks in our SOW: Major Task 3 (consecutively numbered from major tasks under Specific Aim 1) was the generation of knock-in strains with mutated T7 trypsinogen and contained four sub-tasks: design and construction of targeting vectors; targeting in mouse embryonic stem cells, production of chimeric mice and production of F1 heterozygous

mutant mice. Major Task 4 was to assess expression levels of mutant trypsinogens in the newly made mouse strains. **MAJOR TASK 3 OF THIS AIM HAS BEEN COMPLETED. ALL STATED GOALS HAVE BEEN MET. See our previous year-1 report for detailed description of accomplishments. MAJOR TASK 4 HAS BEEN PARTIALLY COMPLETED.**

On the basis of our results under Specific Aim 1, we selected three mutations for further analysis

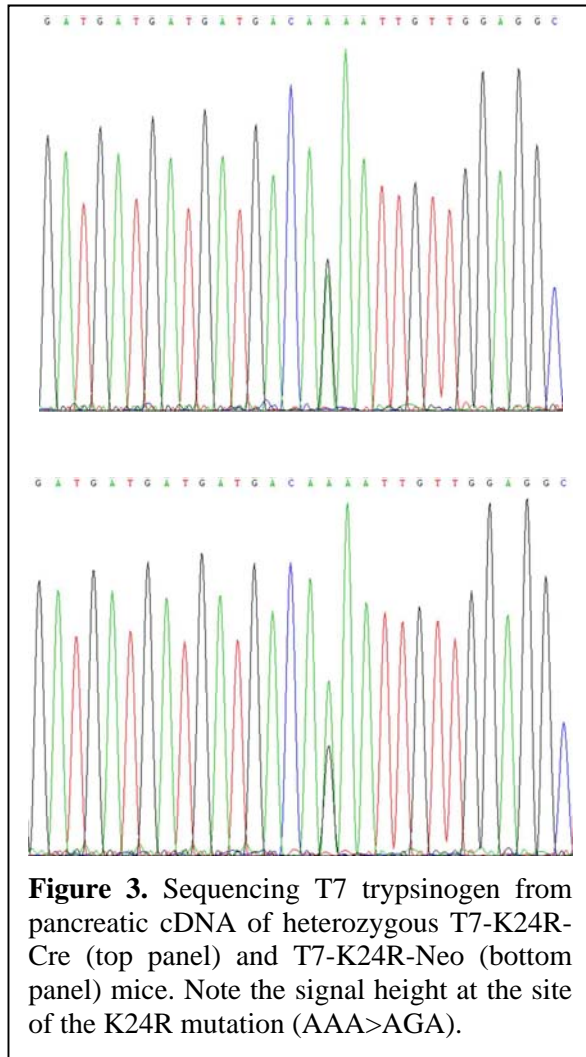
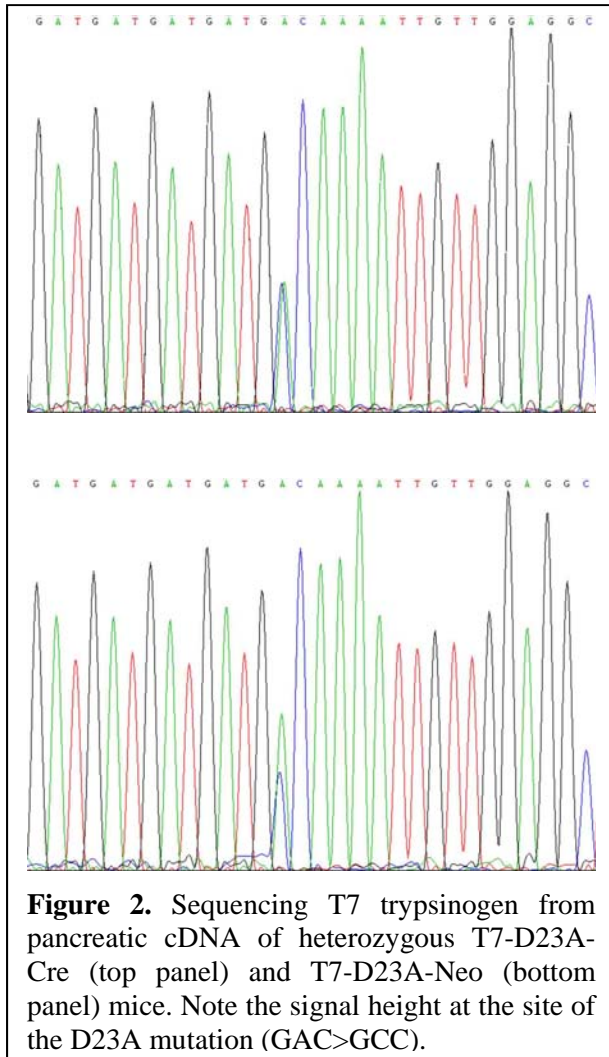


in the mouse: D23A, D23del and K24R. The T7 gene contains five exons and all mutations were targeted to exon 2 using homologous recombination in embryonic stem (ES) cells. As shown in Figure 1, the recombined allele contains a neomycin (Neo) cassette to allow for positive selection of targeted ES cells. The neomycin gene is flanked by loxP sites which was used to remove the cassette by breeding with mice expressing the Cre recombinase in the mouse embryo (Cre-deleter strain). The offspring of this mating had the neomycin cassette excised in all tissues including the germline. Overall six strains were generated, T7-D23A-Neo, T7-D23del-Neo, T7-K24R-Neo, T7-D23A-Cre, T7-D23del-Cre and T7-K24R-Cre. The Neo designation indicates that the strain still contains the neomycin cassette, whereas the Cre designation indicates that the strain has been bred with the Cre-deleter strain and the neomycin cassette is removed. All

mice are on the C56BL/6N substrain background. Note that even after removal of the neomycin cassette a “scar sequence” corresponding to a single loxP site is left behind in intron-1. As discussed below, this did not cause any expression problems in our case. On the other hand, the presence of the neomycin cassette interfered with mRNA expression and all three T7-Neo strains expressed about half as much T7 trypsinogen as the T7-Cre strains.

Assessing expression levels of mutant trypsinogens (Specific Aim 2, Major Task 4).

To estimate mRNA expression of the mutant trypsinogens, we used heterozygous mice and prepared cDNA from their pancreas. Sanger-sequencing of the cDNA samples with primers specific for T7 trypsinogen demonstrated the presence of two overlapping signals at the site of the mutation. Judged by the signal height, expression of wild-type and mutant alleles were comparable in the T7-D23A-Cre and T7-K24R-Cre mice. On the other hand, the mutant signal was about half the size of the wild-type signal in the T7-D23A-Neo and T7-K24R-Neo mice (Figures 2 and 3). Similar analysis of the T7-D23del-Cre and T7-D23del-Neo mice are underway at the time of writing this report.



To verify expression of mutant trypsinogens at the protein level, we will perform chromatographic analysis of the total trypsinogen fraction isolated from mouse pancreata (Subtask 4a). This subtask has been delayed until homozygous animals could be generated. Homozygous animals are now available and chromatographic analysis will be performed in the final year of the grant.

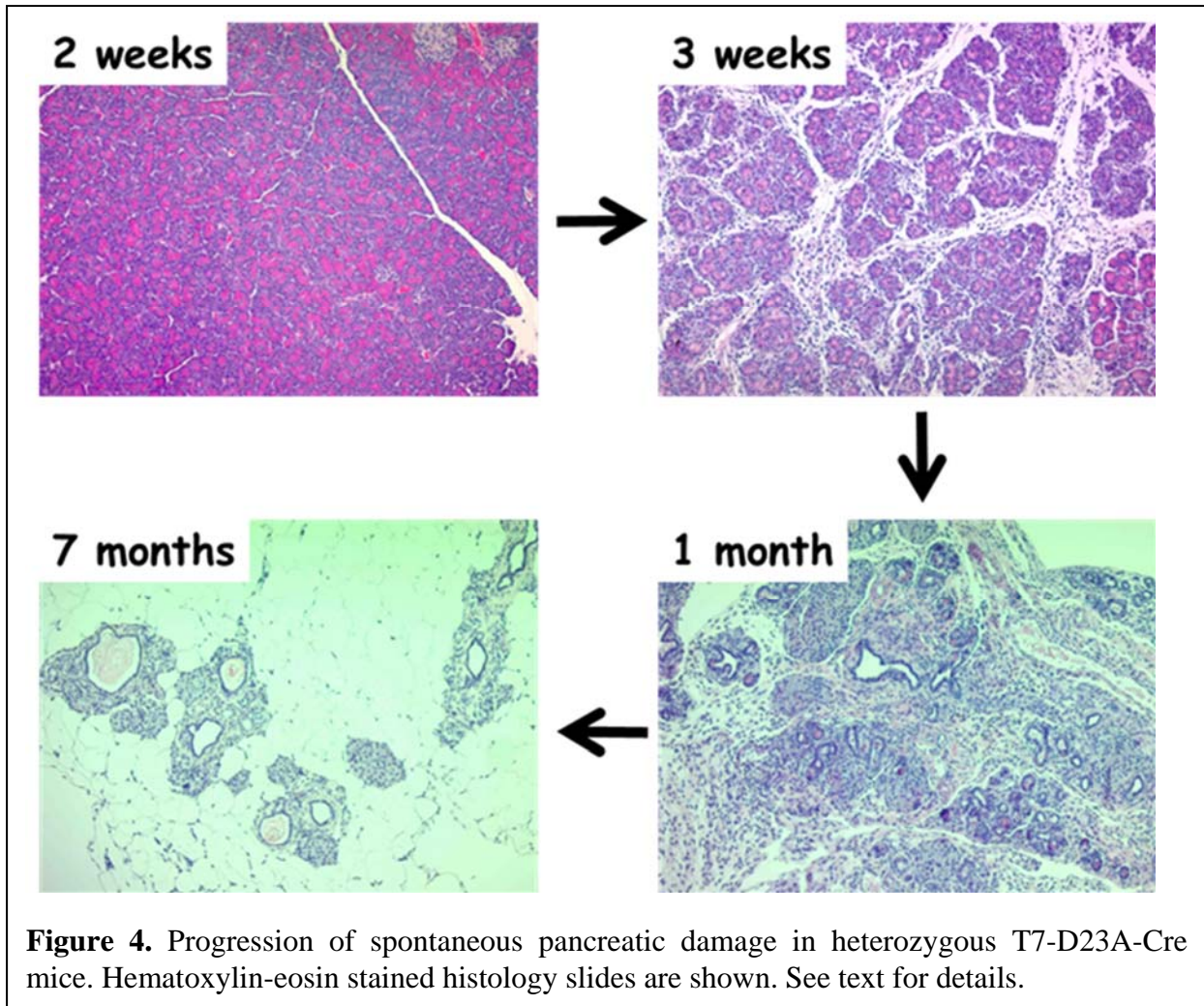
SUMMARY OF RESULTS FOR SPECIFIC AIM 2. We successfully created knock-in mouse strains with mutated T7 trypsinogen. The mutant trypsinogens are expressed as well as the wild-type at the mRNA level in the T7-Cre strains. In the T7-Neo strains which also carry a neomycin cassette in intron-1 of the T7 gene, mRNA expression of the mutant allele is about half of the wild-type levels.

Specific Aim 3. Characterize the newly created mouse strains with respect to spontaneous pancreatitis and sensitivity to experimentally induced pancreatitis.

Under this aim, we had two major tasks in our SOW: Major Task 5 was to assess spontaneous pancreatitis in mutant mice while Major Tasks 6 and 7 were to assess increased susceptibility to

acute and chronic pancreatitis in mutant mice. We made significant progress with respect to Major Task 5, whereas Major Tasks 6 and 7 will be executed in the third year of the grant.

The most **stunning observation** from these studies is that heterozygous T7-D23A-Cre mice develop spontaneous pancreatic damage and features of chronic pancreatitis starting at the age of 3 weeks (Figure 4). Histologically (H&E staining) the pancreas of 2-week old mice looks normal while at 3 weeks of age acini show a more disorganized architecture with acinar cell atrophy and dropout and inflammatory cell infiltration. This pancreatitis-like condition is progressive as at 4 weeks there is hardly any normal acini observed and regenerative tubular complexes appear in abundance, some of these form large distorted duct-like structures filled with eosinophilic,



presumably precipitated, material. There is visible accumulation of extracellular material; suggestive of fibrosis characteristic of chronic pancreatitis. Fibrosis gets replaced eventually by fatty infiltration as pancreata of older mice (7 months) are significantly atrophic and contain mostly adipocytes. The penetrance of pancreatic damage is 100% and the age of onset is consistently 3-4 weeks so far. Remarkably, despite the apparent destruction of their pancreas these mice do well and show no signs of distress. They are somewhat smaller than their wild-type littermates but gain weight with similar kinetics and breed normally. Interestingly, T7-D23A-Neo mice, which express lower levels of the mutant trypsinogen, exhibit pancreatic

damage at a later age (>3 months) and age of onset shows more variability. In contrast to T7-D23A-Cre, heterozygous mutant mice with the D23del or K24R mutation show no spontaneous pancreatitis. We just recently obtained homozygous animals for these strains and analysis of spontaneous pancreas damage is underway at the time of writing this report.

SUMMARY OF RESULTS FOR SPECIFIC AIM 3. We successfully created a mouse strain which exhibits features of human hereditary pancreatitis. Mice with the D23A mutation in their T7 trypsinogen will **develop spontaneous, progressive pancreatitis** with eventual loss of acinar cells and preservation of islets (Major Task 5). More detailed characterization of this exciting strain will be performed in the third year of grant. Similarly, Major Tasks 6 and 7, designed to assess increased susceptibility to acute and chronic pancreatitis in mutant mice which do not develop spontaneous disease (strains T7-D23del-Cre, T7-D23del-Neo, T7-K24R-Cre and T7-K24R-Neo), will be accomplished in the third year.

What opportunities for training and professional development has the project provided?

With respect to “training”, all project personnel have been continuously mentored by the PI utilizing one-on-one meetings, email exchanges and weekly group discussions. These interactions focused on the project from conceptual design to relevant technical details. With respect to “professional development”, all project personnel attended departmental seminars on a regular basis (10-12 seminars in past funding year).

How were the results disseminated to communities of interest? Results from the T7-D23A-Cre mouse strain were discussed for the first time in an oral presentation by the PI at the 48th meeting of the European Pancreatic Club in Liverpool, UK, July 6-9, 2016.

What do you plan to do during the next reporting period to accomplish the goals?

We are exactly on track with our originally planned SOW and we will continue our research according to plan. In the final third year of the grant we will focus on Specific Aim 3; continue with more detailed characterization of the spontaneous pancreatitis in T7-D23A-Cre and T7-D23A-Neo mice (Major Task 5) and carry out the experiments related to Major Tasks 6 and 7 designed to assess increased susceptibility to acute and chronic pancreatitis in mutant mice which do not develop spontaneous disease; i.e. strains T7-D23del-Cre, T7-D23del-Neo, T7-K24R-Cre and T7-K24R-Neo.

4.IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Although the project is not complete yet, we can already state that we succeeded generating a mouse model for human hereditary pancreatitis, which was never before accomplished in the field of pancreatitis research. Our model exhibits spontaneous, progressive pancreatitis resulting in almost complete pancreas destruction while endocrine islets are preserved. This new model will allow more detailed mechanistic studies on the role of trypsin-induced cell death and inflammation in pancreatitis and facilitate preclinical testing of interventions designed to cure or prevent pancreatitis, particularly those targeted to trypsin.

What was the impact on other disciplines? Nothing to report.

What was the impact on technology transfer? Nothing to report.

What was the impact on society beyond science and technology? Nothing to report.

5.CHANGES/PROBLEMS:

Changes in approach and reasons for change. No changes in approach to report.

Actual or anticipated problems or delays and actions or plans to resolve them. No problems or delays were encountered; we have made excellent overall progress.

Changes that had a significant impact on expenditures. Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Significant changes in use or care of human subjects. No change to report.

Significant changes in use or care of vertebrate animals. No change to report.

Significant changes in use of biohazards and/or select agents. No change to report.

6.PRODUCTS:

Publications, conference papers, and presentations. Nothing to report.

Journal publications. Nothing to report.

Books or other non-periodical, one-time publications. Nothing to report.

Other publications, conference papers, and presentations. Results from the T7-D23A-Cre mouse strain were discussed for the first time in an oral presentation by the PI at the 48th meeting of the European Pancreatic Club in Liverpool, UK, July 6-9, 2016.

Website(s) or other Internet site(s). Nothing to report.

Technologies or techniques. Nothing to report

Inventions, patent applications, and/or licenses. Nothing to report

Other Products. The novel strains that develop spontaneous pancreatitis should serve as important models for the study of human pancreatitis, hereditary pancreatitis in particular.

7.PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: **Miklos Sahin-Toth, M.D., Ph.D.**

Project Role: PI

Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0003-4513-9922

Nearest person month worked: 2 months

Contribution to Project: PI, responsible for all aspects of project.

Funding Support: This grant.

Name: **Andras Szabo, Ph.D.**

Project Role: Research Assistant Professor

Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0001-8687-470X

Nearest person month worked: 1 month

Contribution to Project: Dr. Szabo worked on Specific Aim 2 of the project.

Funding Support: This grant.

Name: **Andrea Geisz, Ph.D.**

Project Role: Post-doctoral Associate
 Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0003-0971-6876
 Nearest person month worked: 10 months
 Contribution to Project: Dr. Geisz worked on all Specific Aims of the project.
 Funding Support: This grant.

Name: **Vera Sahin-Toth**
 Project Role: Laboratory manager
 Researcher Identifier (e.g. ORCID ID): None
 Nearest person month worked: 7 months
 Contribution to Project: Ms. Sahin-Toth provided technical support for all Specific Aims of the project.
 Funding Support: This grant.

Name: **Zsanett Jancso, Ph.D.**
 Project Role: Post-doctoral Associate
 Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0002-0572-5452
 Nearest person month worked: 8 months
 Contribution to Project: Dr. Jancso worked on Specific Aims 2 and 3.
 Funding Support: This grant.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to report. No change in active support.

What other organizations were involved as partners? Nothing to report.

8.SPECIAL REPORTING REQUIREMENTS
COLLABORATIVE AWARDS: Not applicable.
QUAD CHARTS: Not applicable.

9.APPENDICES: None.